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Taxonomic Study of Japanese *Deparia petersenii* (Woodsiaceae) Based on Cytological and Molecular Information

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Deparia petersenii has a large morphological variation in plant size and leaf shape in Japan. To reveal possible distinct biological units of this species, we determined the chromosome numbers and nucleotide sequences of the *rbcL* and *ndhF* genes of 58 plant samples from 16 different localities in Japan. We found three cytotypes (tetraploid, hexaploid, and pentaploid) and ten cpDNA haplotypes in Japanese *D. petersenii*. The cpDNA haplotypes of the tetraploid were different from those of the hexaploid, thus genetically differentiating these two cytotypes. Morphological differentiation was also observed between the two cytotypes. As a result, we recognized two distinct biological units in Japanese *D. petersenii*.

Key words: chromosome number, *Deparia petersenii*, *ndhF*, ploidy, *rbcL*

According to a monograph on the genus *Deparia* by Kato (1984), *D. petersenii* is a species diagnosed by a single morphological characteristic: a flat (not incurved) and laciniate indusia. Its geographic distribution is very wide, covering Old World tropical and subtropical areas. Even in Japan, the distribution ranges from the Pacific Ocean side of the southern part of Honshu, Shikoku, and Kyushu islands to the Ryukyu Islands, which extend south toward Taiwan. Both the plant size and the leaf shape of this species are highly variable. Even within Japan, a large degree of morphological variation is observed in this species (Fig. 1).

Ohba (1965), Serizawa (1973), and Kato (1984) taxonomically studied a Japanese *D. petersenii* complex (Ohba and Serizawa called it *Lunathyrium petersenii*). Ohba (1965) divided *L. petersenii* into three varieties: *L. petersenii* var. *petersenii*, var. *grammitoides*, and var. *itoanum*. *Lunathyrium petersenii* var. *petersenii sensu* Ohba (1965) has a lamina width of 5 to 20 cm, and its leaves are oblong-lanceolate to ovate-lanceolate, with deltoid or obtuse pinna apices. This is the most common of the three varieties in Japan. Var. *grammitoides* has a lamina width of less than 3 cm with obtuse pinna apices, and its distribution range is limited to the Pacific Ocean side of the Honshu, Shikoku, and Kyushu islands, and to the Ryukyu Islands. Ohba (1965) combined *Diplazium grammitoides* form. *yakusimense* into this variety. Var. *itoanum* is more than 20 cm in lamina width, and its leaves are oblong-lanceolate to deltoid, with acute pinna apices. This variety is recorded only by its type specimen collected from Yaku Island (*H. Ito s.n.*, 1937 in TI).

Serizawa (1973) recognized two varieties in

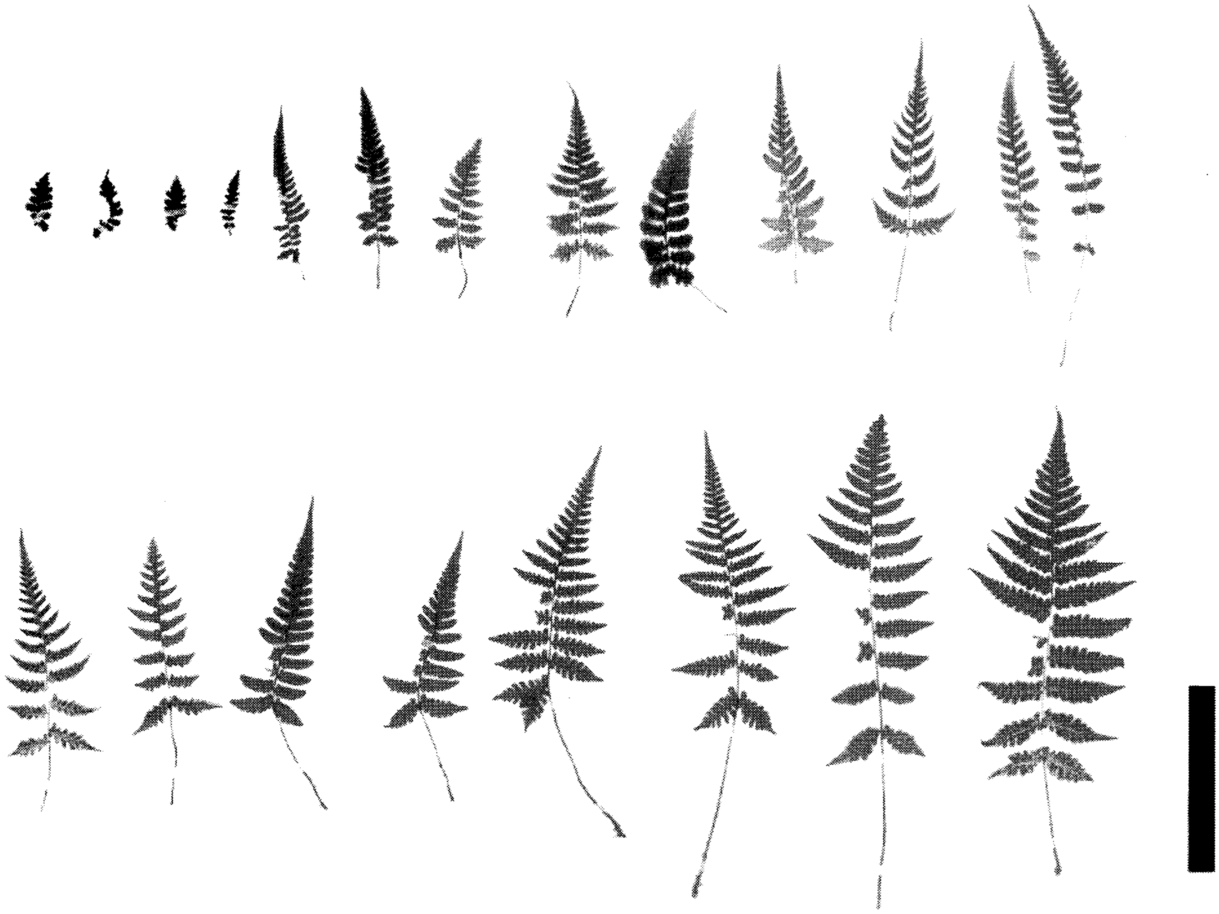

FIG. 1. Variation of leaf size and shape in Japanese *Deparia petersenii*. Scale bar = 10 cm.

Lunathyrium petersenii, var. *petersenii* and var. *yakusimense*. The diagnostic characteristics of var. *yakusimense* are the linear to linear-lanceolate leaves 7 to 25 cm in lamina length and 1.5 to 3.5 cm in lamina width, and the ovate-oblong and almost entire pinnae. This variety grows only on Yaku Island and then only at high elevation. However, Serizawa (1979) combined *L. petersenii* var. *yakusimense* into *Deparia conilii* because he considered var. *yakusimense* to resemble *D. conilii* morphologically as much as it does *D. petersenii*.

Kato (1984) recognized two varieties in *Deparia petersenii* var. *petersenii* and var. *yakusimensis*. Kato (1984) divided *Lunathyrium petersenii* var. *grammitoides sensu* Ohba (1960) into two groups, *D. confluens* and *D. petersenii* var. *yakusimensis*. Kato (1984) categorized *Diplazium grammitoides* into one of the synonyms of *D. confluens* that he treated as a rheophytic species from Southeast Asia (Philippines, Borneo, Java, Bali, Sumbawa, Ternate, Celebes, Fiji, Samoa, Tahiti). On the other hand, he combined *Di. grammitoides* form. *yakusimense* and *L. petersenii* var. *yakusimense sensu* Serizawa (1973) into *D. petersenii* var. *yakusimensis*, which is distributed only in Japan (Kyushu and Shikoku) (Type: *Masamune s.n.*, 1928 in TI). Kato (1984) also mentioned that, although many small individuals grow at both low and high elevations on Yaku Island, the small plants growing at low elevations (< 500 m) are juvenile individuals of var. *petersenii*, while the plants growing at higher elevations (> 500 m) are var. *yakusimensis*.

Very little information is available on the *Deparia petersenii* complex, especially regarding its genetic background. As for cytological information, Kurita (1960, 1967) reported a tetraploid (4x) cyto-

type from Japanese *Lunathyrium petersenii* var. *petersenii sensu* Ohba (1965). Kurita (1972) reported a hexaploid (6x) cytotype from *L. petersenii* var. *grammitoides sensu* Ohba (1965). However, each of these cytological findings was obtained from only one individual. Therefore, more detailed cytological information on *D. petersenii* is necessary to understand the relationships between the wide morphological and cytological variations within this species.

In the present study, we collected plant samples from 16 different localities, covering the entire range of both intraspecific morphological variation and geographical distribution in Japan (Fig 2). We planted these samples in our greenhouse to observe their morphological stability. Subsequently, we used these samples to make cytological (chromosome number) and molecular analyses to reveal the genetic properties of Japanese *Deparia petersenii*. Intraspecific polyploidy is sometimes related to size variation in plants (Müntzing 1936). In pteridophytes, for example, Masuyama (1979) reported the existence of diploid (2x) and tetraploid (4x) plants in *Phegopteris decursive-pinnata*, and that the leaf size of the 4x plants was smaller than that of the 2x. It is possible that ploidy level varies in *D. petersenii*, and that at least some degree of plant size variation in this species may be related to such an intraspecific polyploidy. Thus, we cytologically examined many individuals of *D. petersenii* in this study.

Recently, molecular information, especially DNA sequence data (e.g. *rbcL* sequences), have provided powerful clues to reveal cryptic species of various ferns such as *Hymenasplenium obliquissimum* (Aspleniaceae), *Diplazium doederleinii* (Woodsiaceae), *Ceratopteris thalictroides* (Parkeriaceae), *Cheiropleuria bicuspis* (Dipteridaceae), and *Asplenium nidus* (Aspleniaceae) (Murakami *et al.* 1998a, 1998b, Takamiya *et al.* 1999, Kato *et al.* 2001, Yatabe *et al.* 2001, Masuyama *et al.* 2002). Hasebe *et al.* (1995) and Sano *et al.* (2000a, b, c) determined the *rbcL* sequences of 19 species of *Deparia* including *D. petersenii*. Sano *et al.* (2000c) reported that they could not find a sufficient sequence variation to solve the phylogenetic relationship between different species of *Deparia*. Therefore, in the present study we developed new primers for PCR amplification of the *ndhF* gene, which has a higher molecular evolutionary rate than *rbcL* in angiosperms (Olmstead & Sweer 1994). As a result, we found sequence variations in *rbcL* and *ndhF* genes in *D. petersenii*. We also examined relationships among cytotypic, morphological and DNA haplotypic variations in this species.

Materials and methods

Plant materials

A total of 58 individuals of *Deparia petersenii* from 16 localities in Japan were collected for cytological and molecular analyses. Information on the collection sites, vouchers, and chromosome numbers appears in Table 1. Voucher specimens were deposited in the herbarium of the Graduate School of Science, Kyoto University (KYO). Living stocks were cultivated in the greenhouse of the Botanical Garden, Graduate School of Science, Kyoto University. We mapped all recognizable sporophytes of *Deparia petersenii* in a 10×6 m area along the Kusukawa trail on Yaku Island. All mapped individuals were collected for cytological, molecular, and morphological analyses.

Cytological analysis

For observations of mitotic chromosomes, we employed the techniques described by Takamiya (1993). Root tips were pretreated with 0.002 M 8-hydroxyquinoline for 6 hr at about 20°C. After fixation in 45% acetic acid, the root tips were hydrolyzed in 1 M HCl at 60°C for 2 min and then squashed in 2% aceto-orcein. For examinations of spore viability, gametophytes were cultured from spores collected from an individual of *Deparia*

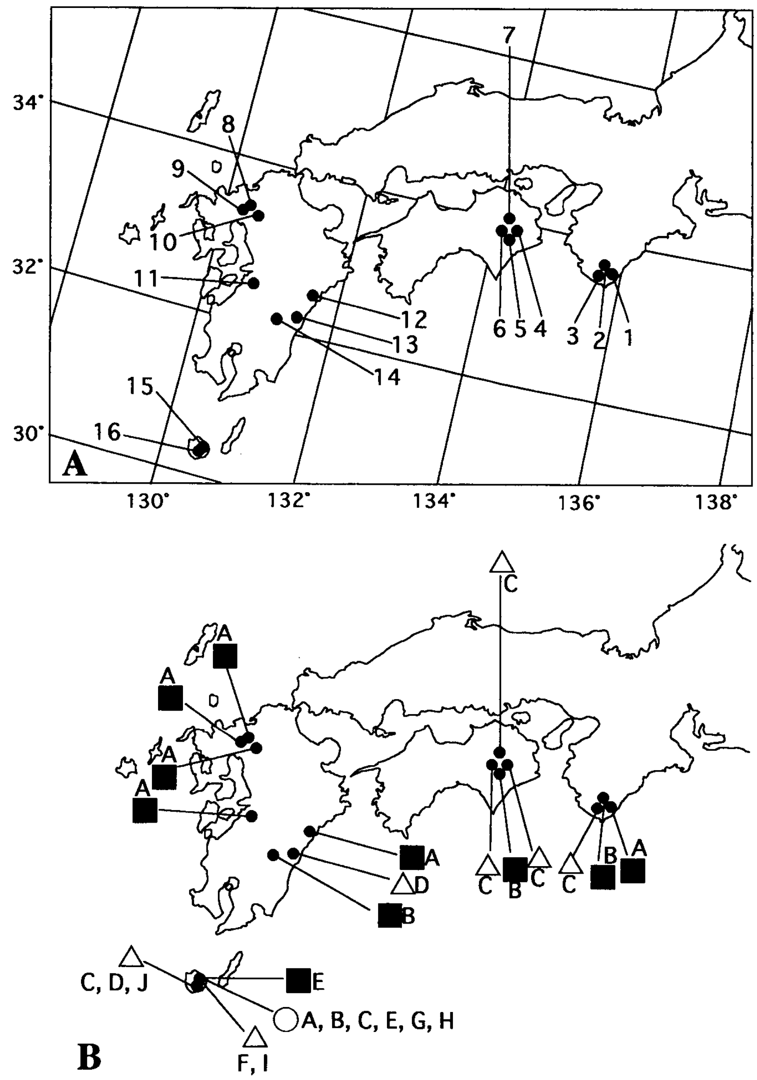

FIG. 2. Localities of plant materials examined in this study. A. Map showing collection sites (solid circles). Exact localities and corresponding numbers are given in Table 1. B. Geographical distributions of three cytotypes and ten cpDNA haplotypes (A-J). The sources and data of the haplotypes are shown in Tables 1 and 2. Square = tetraploid, Triangle = hexaploid, Circle = pentaploid.

petersenii (voucher: *Shinohara 20118025*). The gametophyte cultivation followed the method of Ohta & Takamiya (1999).

rbcL *and* ndhF *Sequencing*

DNA data were obtained from the same materials that were used for the cytological analysis. Total DNA was extracted from fresh leaves using the CTAB extraction method (Doyle & Doyle 1987). Double-stranded DNAs of two chloroplast genes (*rbcL* and *ndhF*) were amplified by 35 cycles of symmetric polymerase chain reaction (PCR). The universal primers designed by Hasebe *et al.* (1994) were used for amplification of *rbcL*. For PCR amplification of the *ndhF* gene, we developed a pair of new primers (ATCGGTATTTATG-GTCTTGG and CCATCGATGATCCATTGATC) by comparing the sequences of the *ndhF* region of *Nicotiana tabacum*, *Osumunda japonica*, and *Lycopodium serratum* obtained from a DNA database. PCR products were purified by electrophoresis in 1.0% agarose gel using 1x TAE buffer. The

TABLE 1. Voucher information of plant samples and data of cytological and molcular analyses.

Population. no	Locality	Voucher*	Chromosome no. (2*n*)	Ploidy	cpDNA haplotype
1	Kinokawa, Singu City,	S. 20118055	160	4*x*	A
	Wakayama Pref.	S. 20118056	160	4*x*	A
2	Deai, katuura-cho, Wakayama Pref.	S. 20118005	160	4*x*	B
3	Nagai, Wakayama Pref.	S. 20118050	240	6*x*	C
4	Aioi-cho, Tokushima Pref.	S. 20118062	160	4*x*	B
		S. 20118063	160	4*x*	B
		S. 20118064	160	4*x*	B
5	Jintu, Aioi-cho, Tokushima Pref.	S. 20118058	240	6*x*	C
		S. 20118049	240	6*x*	C
6	Byoudouji, Tokushima Pref.	S. 20118054	240	6*x*	C
7	Kakurinji, Tokushima Pref.	S. 20118048	240	6*x*	C
8	Nisikuma, Fukuoka Pref.	S. 20118052	160	4*x*	A
9	Myoukenndaki, Fukuoka Pref.	S. 20118008	160	4*x*	A
10	Amitori, Fukuoka Pref.	S. 20118006	160	4*x*	A
11	Kiharayama, Kumamoto Pref.	S. 20118007	160	4*x*	A
12	Hasugaike, Miyazaki Pref.	S. 20118047	160	4*x*	A
13	Nichinan City, Miyazaki Pref.	S. 20118053	240	6*x*	D
		S. 20118003	240	6*x*	D
		S. 20118051	240	6*x*	D
14	Fujikawachi, Miyazaki Pref.	S. 20118045	160	4*x*	B
		S. 20118046	160	4*x*	B
		S. 20118060	160	4*x*	B
		S. 20118061	160	4*x*	B
15	Kusukawa, Yaku Island,	S. 20118010	240	6*x*	F
	Kagosima Pref.	S. 20118012	240	6*x*	F
		S. 20118014	240	6*x*	F
		S. 20118015	240	6*x*	F
		S. 20118016	240	6*x*	I
		S. 20118017	240	6*x*	F
		S. 20118018	240	6*x*	F
		S. 20118019	200	5*x*	B
		S. 20118020	240	6*x*	F
		S. 20118021	200	5*x*	A
		S. 20118022	200	5*x*	G
		S. 20118023	160	4*x*	E
		S. 20118024	200	5*x*	B
		S. 20118025	200	5*x*	E
		S. 20118026	240	6*x*	F
		S. 20118027	240	6*x*	I
		S. 20118028	200	5*x*	H
		S. 20118029	200	5*x*	C
		S. 20118030	160	4*x*	E
		S. 20118031	240	6*x*	F
		S. 20118034	240	6*x*	F
		S. 20118035	240	6*x*	F
		S. 20118036	200	5*x*	A

TABLE 1. continued

Population. no	Locality	Voucher*	Chromosome no. ($2n$)	Ploidy	cpDNA haplotype
		S. 20118037	160	$4x$	A
		S. 01091206	240	$6x$	C
16	Kosugitani, Yaku Island, Kagosima Pref.	S. 01091435	240	$6x$	J
		S. 01091413	240	$6x$	C
		S. 01091436	240	$6x$	J
		S. 01091434	240	$6x$	J
		S. 01091437	240	$6x$	J
		S. 01091412	240	$6x$	C
		S. 01091410	240	$6x$	D
		S. 01091419	240	$6x$	D
		S. 01091417	240	$6x$	C
		S. 01091418	240	$6x$	C

* S.: Shinohara

TABLE 2. Ten cpDNA haplotypes in Japanese *Deparia petersenii*. (Numbers indicate base substitution sites)

cpDNA haplotype	*rbcL* 847	930	976	1084	DDBJ No.	*ndhF* 1191	1813	1887	DDBJ No.	Polyploidy	
A	C	C	G	G	AB095977	C	A	C	AB095980	$4x$	$5x$
B	C	C	G	G	AB095977	T	C	C	AB095981	$4x$	$5x$
C	C	C	A	A	AB095976	C	A	C	AB095980	$5x$	$6x$
D	C	C	A	A	AB095976	C	A	G	AB095982	$6x$	
E	T	C	G	G	AB095974	T	A	C	AB095979	$4x$	$5x$
F	T	C	A	A	AB095975	C	A	C	AB095980	$6x$	
G	T	C	G	G	AB095974	T	C	C	AB095981	$5x$	
H	C	C	A	A	AB095976	T	C	C	AB095981	$5x$	
I	T	C	A	A	AB095975	T	C	C	AB095981	$6x$	
J	C	T	A	A	AB095978	C	A	C	AB095980	$6x$	

amplified DNA fragments were eluted from the gel using the GENECLEAIII kit (BIO101). Purified PCR products were sequenced in both directions using a Big Dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) on an Applied Biosystems Model 377 automated sequencer (Perkin-Elmer Applied Biosystems). The sequences obtained were aligned using Sequence Navigator Software (Perkin-Elmer Applied Biosystems). The DDBJ accession numbers for the nucleotide sequences of haplotypes are shown in Table 2.

Morphological analysis

The morphological characteristics of voucher specimens used for cytological and molecular analyses were measured quantitatively. We measured each sample's lamina length and width. The morphological differences between ploidies or cpDNA haplotypes were statistically tested by t-test. To test for differences between cpDNA haplotypes, only those haplotypes that appeared in five or more individuals within the population were tested.

Results

Cytological Observations

Three cytotypes were found in *Deparia petersenii*. Eighteen plants collected from ten localities had

$2n$ = 160 chromosomes, indicating that they were tetraploid ($4x$) based on x = 40 (Figs. 3, 6). Thirty-five plants from seven localities were hexaploid ($6x$), with $2n$ = 240 chromosomes (Figs. 5, 8). Eight plants, all from a single location (Yaku Island), were pentaploid ($5x$) with $2n$ = 200 (Table 1, Figs. 4, 7). In these $5x$ plants, fertile leaves were found in only one of the eight individuals. The spores collected from this single $5x$ plant (voucher: *Shinohara 20118025*) did not germinate at all.

Although the geographical distribution ranges of the $4x$ and $6x$ types overlapped, these two cytotypes were not found together within the same population, except in the Yaku Island population (Table 1, Fig. 2). Either $4x$ or $6x$ plants were found in the geographical range from Wakayama prefecture to Kagoshima prefecture. On Yaku Island (Kagosima prefecture), all three cytotypes ($4x$, $5x$, $6x$) were sympatrically found in a single population (Kusukawa) (Table 1, Fig. 9A).

DNA variation

We sequenced 1,236 nucleotides of the *rbcL* gene and 838 nucleotides of the *ndhF* gene for all of the plant materials obtained. Seven variable sites (four in *rbcL* and three in *ndhF*) were found, and ten haplotypes in total were recognized. Types D, F, I, and J were found only in the $6x$ plants. Types A, B, and E were found only in the $4x$ and $5x$ plants. Type C was found in the $5x$ and the $6x$ plants. Types G and H were found only in the $5x$ plants on Yaku Island. Thus, no haplotype was shared between the $4x$ and $6x$ plants. Based on these molecular findings, $4x$ and $6x$ plants were genetically differentiated even in plastid DNA. With regard to geographical distribution, types A, B, and C were widely distributed in Japan (Fig. 2). Four of the ten haplotypes (types F, G, H, and I) were restricted to Yaku Island. In the Kusukawa population on Yaku Island, eight of the ten haplotypes were sympatrically growing (Table 1, Fig. 9B).

Comparison of qualitative and quantitative morphological characteristics between the three cytotypes

The $4x$ and $6x$ plants of *Deparia petersenii* were morphologically distinguishable. The leaves of the $4x$ type were ovate-lanceolate or narrowly deltoid in shape and deeply bipinnatifid, whereas those of the $6x$ type were linear-lanceolate and shallowly bipinnatifid. Quantitative characteristics were as follows. The $4x$ plants were larger in size than the $6x$ plants (Table 3); lamina length and width were significantly larger in the $4x$ plants than the $6x$ ($P < 0.01$). The $5x$ plants had an intermediate leaf morphology and ranked in size between the $4x$ and the $6x$. The lamina length and width of the $5x$ plants differed significantly from those of the $4x$ or the $6x$ ($P < 0.05$). Greater size-variation was observed among the $4x$ plants than among the $6x$ plants. No significant leaf size difference was observed between every two haplotypes found in the same ploidy level (Table 4).

TABLE 3. Quantitative morphological characters by ploidy.

Character	Tetraploid (N=18)	Pentaploid (N=8)	Hexaploid (N=32)
Lamina length (cm)	10.87±4.12	9.91±3.39	4.18±1.77
	(5.5-19.8)	(5.5-15.2)	(2.3-8.8)[a]
Lamina width (cm)	4.80±2.55	3.06±0.55	1.57±0.59
	(1.1-11)	(2.2-3.7)	(0.8-3.2)

a Mean±s.d. (Min.-Max.)

Mapping analysis at Kusukawa population on Yaku Island

We performed a individual mapping at Kusukawa on Yaku Island (Fig. 9A, B). At the mapped site, 3 tetraploids, 8 pentaploids, and 14 hexaploids were recorded. The $4x$ and $5x$ plants grew in both the uphill and downhill portions of the site. For the $4x$ plants, the same number of individuals grew in both the uphill and downhill portions of the site. All of the $6x$ individuals that had smaller leaves grew only in the lower portion of the site (Fig. 9A).

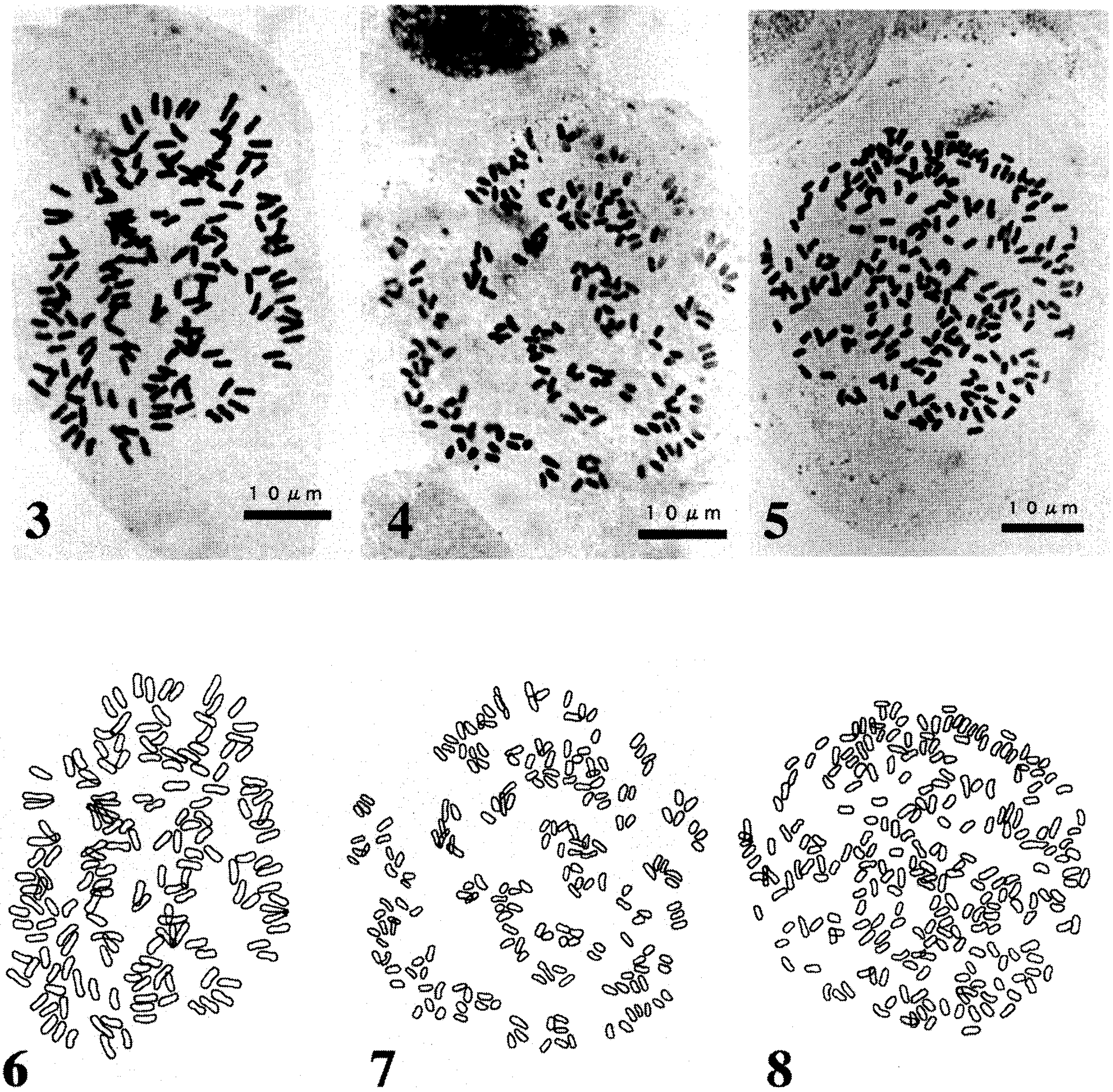

FIG. 3-8. Somatic metaphase chromosomes of *Deparia petersenii* and explanatory drawings. 6, $2n = 160$. 7, $2n = 200$. 8, $2n = 240$. Scale bar = 10μm. 3, 6, $2n = 160$. 4, 7, $2n = 200$. 5, 8, $2n = 240$. Scale bars = 10μm.

TABLE 4. Quantitative morphological characters by haplotype.

Character	A (N=10)	B (N=10)	C (N=11)	D (N=5)	E (N=3)	F (N=11)	G (N=1)	H (N=1)	I (N=2)	J (N=4)
Lamina length (cm)	11.0±2.24	10.57±5.06	4.84±3.91	4.0±2.70	8.80±4.33	4.04±1.37	5.50	12.20	5.85±1.91	5.03±1.35
	(7.3-14.2)[a]	(6.1-19.8)	(2.5-15.2)	(2.3-8.8)	(5.5-14)	(2.5-7.2)			(4.5-7.2)	(3.4-6.7)
Lamina width (cm)	4.28±1.70	4.97±3.13	1.45±0.77	1.06±0.28	2.67±0.47	2.03±0.54	3.50	3.30	1.95±0.07	1.08±0.10
	(2.5-8.2)	(1.1-11)	(1-3.6)	(0.8-.14)	(2.2-3)	(1.2-3.2)			(1.9-2)	(1-1.2)

a Mean±s.d. (Min.-Max.)

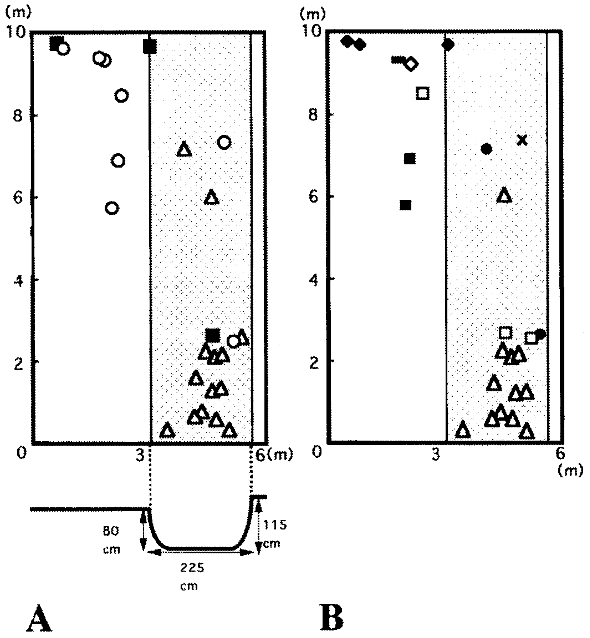

FIG. 9. Sympatric population at the mapping site on Yaku Island. The shaded area on the right side of the site shows a shallow hollow.A. Distribution map by ploidy. Square = tetraploid, Triangle = hexaploid, Circle = pentaploid. B. Distribution map by cpDNA haplotypes. Open square = type A, Solid square = type B, Open diamond = type C, Solid diamond = type E, Open triangle = type F, Cross shape = type G, Bar = type H, Solid circle = type I.

Clumped distribution was also observed for the 6*x* plants at this site. With regard to cpDNA haplotypes, all eight (A, B, C, E, F, G, H, and I) were found at this site. Plants of the same haplotype tended to grow adjacent to each other (Fig. 9B). As for their ecological differentiation, the 6*x* type tended to grow at the bottom of a shallow hollow through which a small stream would form after heavy rain. On the other hand, the 4*x* and 5*x* plants in the sympatric population grew not only at the bottom of the shallow hollow but also on the banks of the river. In allopatric populations in Honshu and Shikoku, the 6*x* type was never found near streams and was often found on sunny roadsides or in the gardens of old temples. The 4*x* type was also found in similar habitats. No clear ecological differentiation was recognized between 4*x* and 6*x* plants of *Deparia petersenii* on the Honshu, Shikoku, or Kyushu islands.

Discussion

Previously, Kurita (1960, 1967) reported a 4*x* (2*n* =160 and *n* = 80) cytotype of *Deparia petersenii* based on his cytological observations of two plants, one collected from Kikugawa, Shizuoka prefecture, the other from Ichikawa City, Chiba prefecture. He also reported a 6*x* (2*n* = 240) cytotype from Yaku Island, Kagoshima prefecture (Kurita 1972). However, his observation was based on only three individuals. Therefore, the geographical distribution of each cytotype has never been analyzed. In this study, we cytologically observed many individuals of *D. petersenii* and found that both the 4*x* and 6*x* plants are widely distributed in Japan. Our study also provided the first record of the 5*x* cytotype from this species. Although the geographical distribution ranges of the 4*x* and 6*x* plants overlapped, these two cytotypes were not found within the same populations, except in the case of Kusukawa population on Yaku Island (Table 1, Figs. 2, 9A). The 5*x* plants were observed only in Kusukawa population on Yaku Island.

A high correlation between ploidy level and leaf size was observed in this study. The 6*x* plants had smaller laminae than the 4*x*, and those of the 5*x* were intermediate between those of the 4*x* and 6*x* plants (Table 3). These size differences among the three cytotypes were stable even after plants were transplanted to a greenhouse (Fig. 10). Therefore, we may conclude that the size differences between polyploids are due not to plasticity but to stable genetic control. Size difference is also effective for phenotypically differentiating the 4*x* from the 6*x* plants in the field. Considering the intermediate lamina size, the sterility of the 5*x* plants, and the fact that some haplotypes (A, B, C and E) of the 5*x* plants were shared with either the 4*x* or 6*x* plants, the 5*x* plants might be F1 hybrids of the 4*x* and 6*x* cytotypes. However, among the 4*x* and 6*x* types, we could not find any possible parents of the two haplotypes that were found only in the 5*x* type. The

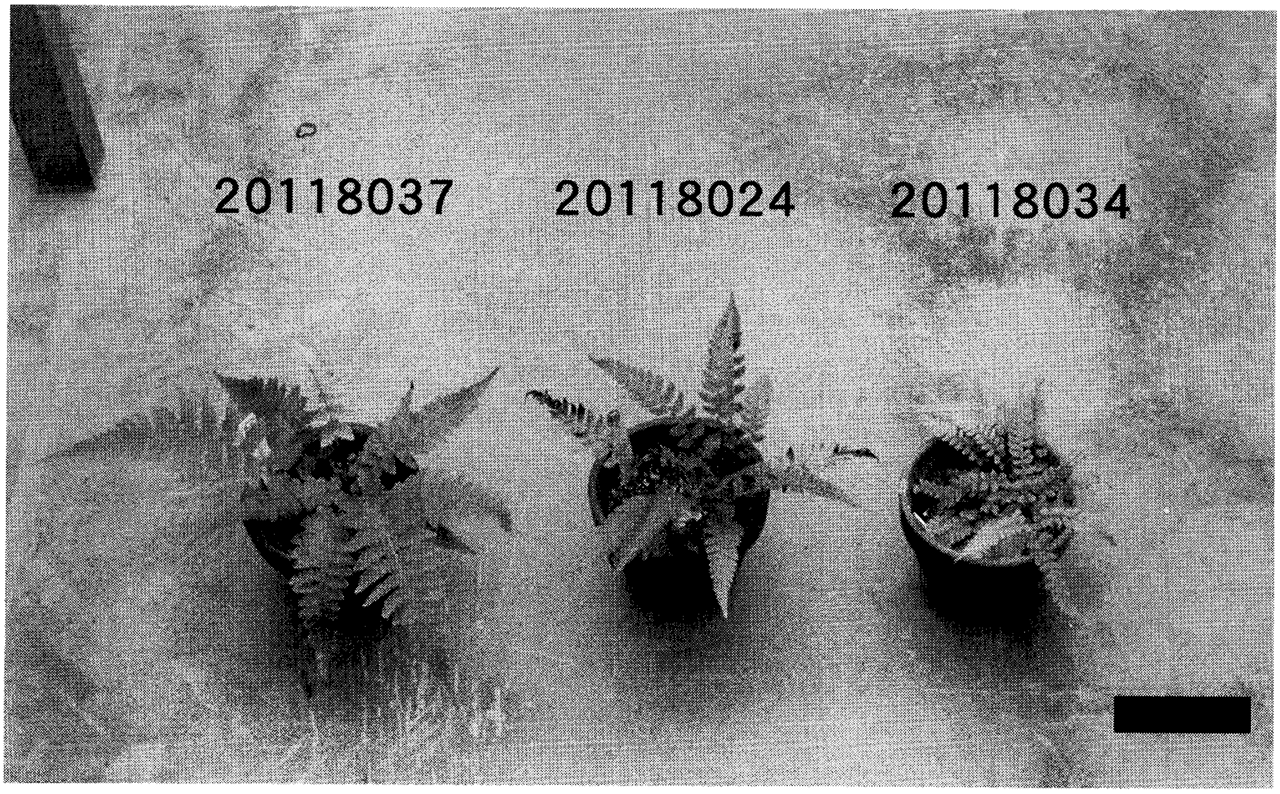

FIG. 10. Gross morphology of the plants of tetraploid (left), pentaploid (center), and hexaploid (right). Scale bar = 10cm.

abundance of *Deparia petersenii* in its wild population increases in the southern Kyushu, and increases markedly on Yaku Island. Our screening of haplotypes in *D. petersenii* might not have produced a sufficient sample size; in a larger sample, these haplotypes found only in the 5*x* plants might be found in either the 4*x* or 6*x*.

We recognized two distinct biological units (the 4*x* and 6*x* plants) in Japanese *Deparia petersenii* based on cytological, molecular, and morphological information. We considered that the type specimen of var. *yakusimensis* (Type: *Masamune s.n.,* 1928 in TI) is in the range of morphological variation of the 6*x* type based on its smaller leaf size and its locality (Yaku Island). Because we collected plant materials from low and high elevation in Yaku Island which covered most of the morphological variation and our plant samples should contained var. *yakusimensis* as well. Therefore, we concluded that the 4*x* and 6*x* plants found in this study are *D. petersenii* var. *petersenii* and var. *yakusimensis*, respectively. In the future, var. *petersenii* and var. *yakusimensis* should be treated as independent species in the taxonomical sense, as long as distinct biological units are considered different taxonomical species. Kato (1984) mentioned that var. *yakusimensis* is distributed only in Kyushu (including Yaku Island) and Shikoku, but the 6*x* plants were found also on the Kii Peninsula.

Kato (1984) concluded that the small individuals growing at low elevations (< 500 m) on Yaku Island were juveniles of *Deparia petersenii* var. *petersenii*. However, the results of the present study clearly show that the small plants are the 6*x* cytotype and, based on cpDNA data, are also genetically differentiated from the larger plants of the 4*x* cytotype. The existence of a hybrid (5*x*) might have prevented the pteridologists from recognizing two distinct biological units within *D. petersenii*. *Deparia petersenii* var. *yakusimensis*, defined by Kato (1984), was reported to grow in Kyushu and Shikoku. The same study mentioned that, especially on Yaku

Island, this variety grows only at high elevations (> 500 m). Although we do not have sufficient data for statistical testing, the haplotype frequencies of the 6*x* plants seem to differ between the lower and higher elevations on Yaku Island. However, we did not find any significant quantitative morphological differences between the haplotypes from the high and low elevations. At present, it is not clear whether two biological units are contained in the 6*x* plants on Yaku Island. Further analyses of the plants on Yaku Island are necessary.

While several nucleotide variations were found on *rbcL* and *ndhF* genes, high degrees of homoplasy were observed among the haplotypes (Table 2). It has been reported that some hot spots exist on the *rbcL* gene (Murakami 1995). Such hot spots sometimes cause a low value on the constancy index (CI) for a molecular phylogenetic tree of closely related species or intraspecific taxa. For example, the molecular phylogenetic tree of the genus *Hymenasplenium* constructed on the basis of the *rbcL* gene showed a low constancy index (CI = 0.582) (Murakami 1995). Although no information is available on the hot spots of the *ndhF* gene, the high degree of homoplasy found in *D. petersenii* might be attributable to the existence of hot spots in both the *rbcL* and *ndhF* genes.

A high correlation between chromosome numbers and plant sizes has been reported for many plant taxa (Müntzing 1936, Levan 1939, Smith 1939, Lewis 1980). A tetraploid is usually larger than a diploid. However, higher polyploid plants, such as hexaploids or octaploids, are often smaller than diploids or tetraploids in plant size (Müntzing 1936). The comparison between the 4*x* and 6*x* plants in *Deparia petersenii* showed the same trend: the 6*x* plants were smaller than the 4*x* ones. This size difference might be attributable directly to a difference in ploidy level.

Deparia petersenii is distributed in Japan as well as in other areas of East Asia, Southeast Asia, and Oceania. Even larger variations of size and shape have been observed in specimens of *D. petersenii* deposited in herbaria KYO and TI, especially in the specimens collected outside Japan. Therefore, several distinct biological units in *D. petersenii* might yet be found in the distribution ranges outside Japan. Kato (1984) divided the genus *Deparia* into four sections (sect. *Dryoathyrium*, *Lunathyrium*, *Deparia*, and *Athyriopsis*) and put *D. petersenii* into sect. *Athyriopsis*. Most of the species in sect. *Athyriopsis* as defined by Kato (1984) were reported to be polyploid (4*x*, 6*x*) (Takamiya 1996). In sect. *Athyriopsis*, diploids were reported only from *D. otomasui* by Hirabayashi (1970). Therefore, it is very important to search out diploids of sect. *Athyriopsis* in order to clarify the origin and evolution of polyploidy in *D. petersenii* as well as to elucidate the evolutionary history of the section. In order to find diploids and new distinct biological units, we should expand the research area to a wider distribution range of *D. petersenii*.

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References

Doyle, J. J. & J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull. 19: 11-15.

Hasebe, M., T. Omori, M. Nakazawa, T. Sano & M. Kato. 1994. *rbcL* gene sequences provide new evidence for the evolutionary lineages of leptosprangiate ferns. Proc. Natl. Acad. Sci. USA. 91: 5730-5734.

——, P. G. Wolf, K. L. Pryer, K. Ueda, M. Ito, R. Sano, G. J. Gastony, J. Yokoyama, J. R. Manhart, N. Murakami, E. H. Crane, C. H. Haufler & W. D. Hauk. 1995. Fern phylogeny based on *rbcL* nucleotide sequences. Amer. Fern J. 85: 134-181.

Hirabayashi, H. 1970. Chromosome numbers in several species of the Aspidiaceae (2). J. Jpn. Bot. 45: 45-52.

Kato, M. 1977. Classification of *Athyrium* and allied genera of Japan. Bot. Mag. Tokyo 90: 23-40.

——. 1984. A taxonomic study of the Athyrioid fern genus *Deparia* with main reference to the Pacific species. J. Fac. Sci. Univ. Tokyo III, 13: 375-430.

——, Y. Yatabe, N. Sahashi & N. Murakami. 2001. Taxonomic studies of *Cheiropleuria* (Dipteridaceae). Blumea 46: 513-525.

Kurita, S. 1960. Chromosome numbers of some Japanese ferns. J. Jpn. Bot. 35: 269-272 (in Japanese with English summary).

——. 1967. Chromosome numbers of some Japanese ferns (7). Annu. Rep. Foreign Students' Coll. Chiba Univ. 2: 41-56.

——. 1972. Chromosome numbers of some Japanese ferns (8). Annu. Rep. Foreign Students' Coll. Chiba Univ. 7: 47-53.

Levan, A. 1939. Tetraploidy and octoploidy induced by colchicine in diploid *Petunia*. Hereditas 25:109-131.

Lewis, W. H. 1980. Polyploidy in species populations. *In*: Lewis, W. H. (ed.) Polyploidy. pp. 103-144. Plenum Press, New York.

Masuyama, S. 1979. Reproductive biology of the fern *Phegopteris decursive-pinnata* I. The dissimilar mating systems of diploids and tetraploids. Bot. Mag. Tokyo 92: 275-289.

——, S., Y. Yatabe, N. Murakami & Y. Watano. 2002. Cryptic species in the fern *Ceratopteris thalictroides* (L.) Brongn. (Parkeriaceae). I. Molecular analyses and crossing tests. J. Plant Res. 115: 87-97.

Müntzing, A. 1936. The evolutionary significance of autopolyploidy. Hereditas 21: 263-378.

Murakami, N. 1995. Systematics and evolutionary biology of the fern genus *Hymenasplenium* (Aspleniaceae). J. Plant Res. 108: 257-268.

——, J. Yokoyama, X. Cheng, H. Iwasaki, R. Imaichi & K. Iwatsuki. 1998a. Molecular taxonomy of *Hymenasplenium obliquissimum* complex (Aspleniaceae) based on *rbcL* sequence comparisons. Plant Species Biol. 13: 51-56.

——, —— & K. Iwatsuki. 1998b. *Hymenasplenium inthanonense* (Aspleniaceae), a new fern species from Doi Inthanon, and its phylogenetic status. Thai Forest Bull. 26: 40-52.

Ohba, H. 1965. Considerations on the genus *Lunathyrium* in Japan (1). Sci. Rep. Yokosuka City Mus. 11: 48-55.

Ohta, N. & Takamiya, M. 1999. Taxonomic studies of the *Diplazium mettenianum* complex (Woodsiaceae; Pteridophyta) in Japan. Morphology, cytology and taxonomy of the plants with normal-shaped spores. J. Plant Res. 112: 67-86.

Olmstead, R.G. & J. A. Sweer. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. Syst. Biol. 43: 467-481.

Sano, R., M. Ito, S. Kurita & M. Hasebe. 2000a. *Deparia formosana* (Rosenst.) R. Sano as the new name for *Diplazium formosanum* Rosenst. Acta Phytotax. Geobot. 51: 17-20.

——, M. Takamiya, M. Ito, S. Kurita & M. Hasebe. 2000b. Phylogeny of the lady fern group, tribe Physematieae (Dryopteridaecae), based on chloroplast *rbcL* gene sequence. Mol. Phylogenet. Evol. 15: 403-413.

——, ——, S. Kurita, M. Ito & M. Hasebe. 2000c. *Diplazium subsinuatum* and *Di. tomitaroanum* should be moved to *Deparia* according to molecular, morphological, and cytological characters. J. Plant Res. 113: 157-164.

Serizawa, S. 1973. *Lunathyrium japonicum* complex in Japan, Ryukyu and Taiwan. Sci. Rep. Takao Mus. 5: 1-28.

——. 1979. Supplementary notes on the Japanese species of *Deparia* sect. Athyriopsis. J. Jpn. Bot. 54: 178-183.

Smith, H. H. 1939. The induction of polyploidy in *Nicotiana* species and species hybrids by treatment with colchicine. J. Heredity 30: 291-306.

Takamiya, M. 1993. Chromosome numbers of *Woodsia kitadakensis* and *W. subcordata* (Woodsiaceae). J. Jpn. Bot. 68: 73-76.

——. 1996. Index to Chromosomes of Japanese Pteridophyta. (1910-1996). Jap. Pterid. Soc., Tokyo.

——, C. Takaoka & N. Ohta. 1999. Cytological and reproductive studies on Japanese *Diplazium* (Woodsiaceae; Pteridophyta): apomictic reproduction in *Diplazium* with evergreen bi- to tripinnate leaves. J. Plant Res. 112: 419-436.

Yatabe, Y., S. Masuyama, D. Darnaedi & N. Murakami. 2001. Molecular systematics of the *Asplenium nidus* complex from Mt. Halimun National Park, Indonesia; evidence for reproductive isolation among three sympatric *rbcL* sequence types. Amer. J. Bot. 88: 1517-1522.

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